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Silibinin Prevents Lung Tumorigenesis in Wild-type but not in *iNOS*^{-/-} Mice: Potential of Real-time Micro-CT in Lung Cancer Chemoprevention Studies

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Abstract

Purpose—Sustained nitric oxide (NO) generation positively correlates with lung cancer development and progression. Herein, we genetically confirmed this role of *iNOS* and evaluated the chemopreventive efficacy of silibinin in carcinogen-treated B6/129 wild-type (WT) and *iNOS*^{-/-} mice.

Experimental Design—Male B6/129-Nos2^{tm1Lau} (*iNOS*^{-/-}) and B6/129PF2 WT mice were injected i.p. with 1mg/g body weight urethane once weekly for 7 consecutive weeks, followed by silibinin gavage (742mg/kg body weight) for 5 days/week for 18 weeks.

Results—Quantification of micro-CT data in real-time showed that silibinin significantly decreases urethane-induced tumor number and size in WT mice, consistent with measurements made *ex vivo* at study termination. Genetic ablation of *iNOS* decreased urethane-induced tumor multiplicity by 87% ($P < 0.001$) compared to WT mice. Silibinin decreased tumor multiplicity by 71% ($P < 0.01$) in WT mice, but did not show any such considerable effect in *iNOS*^{-/-} mice. Tumors from WT mice expressed more *iNOS* ($P < 0.01$) but almost similar *eNOS* and *nNOS* than those in silibinin-treated mice. In these tumors, silibinin moderately ($P < 0.01$) inhibited cell proliferation but strongly ($P < 0.01$) reduced the number of newly formed nestin-positive microvessels. Silibinin decreased VEGFR2 level, and STAT3 and NF- κ B activation in tumors.

Conclusions—The lack of effect of silibinin in *iNOS*^{-/-} mice suggests that silibinin exerts most of its chemopreventive and angiopreventive effects through its inhibition of *iNOS* expression in lung tumors. Our results support *iNOS* as a potential target for controlling lung cancer, and demonstrate the value of real-time non-invasive micro-CT imaging modality for evaluating the efficacy of lung cancer chemopreventive agents.

Keywords

Chemoprevention; lung cancer; micro-CT; silibinin; *iNOS*

Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States. Lung cancer patients are usually diagnosed at a late stage when prognosis for most patients remains poor, with a 5-year survival rate of less than 16% (1). There has been little improvement in the efficacy of lung cancer treatments in recent decades, indicating the need for alternative strategies to help control this disease (2,3). One way to reduce the risk of this disease is by chemoprevention using phytochemicals administration to high risk populations. A deeper molecular understanding of the events regulating tumor progression in model organisms should enhance development of novel chemopreventive and therapeutic approaches to this problem.

The flavolignan, silibinin, is a major constituent in silymarin, an extract of milk thistle (*Silybum marianum*). Recently, silibinin received significant attention for its strong chemopreventive and anticancer efficacy. Silibinin inhibited growth in cancer models of skin (4,5), prostate (6–9), bladder (10) and colon (11). Silibinin inhibits multiple cytokine induced signaling pathways that regulate inducible nitric oxide synthase (iNOS) expression in A549 cells, and inhibited the *in vivo* growth of A549 xenografts, and reduced the systemic toxicity of doxorubicin in these studies (12,13). Dietary silibinin decreased cell proliferation and angiogenesis by targeting iNOS in a urethane-induced lung tumorigenesis model (14). It has also shown chemotherapeutic efficacy in urethane-induced, established lung adenocarcinomas where it also inhibited angiogenesis (15). Silibinin also reduced proliferation of human non-small cell lung carcinoma (NSCLC) H1299, H460 and H322 cells by targeting cell cycle associated proteins (16).

Silibinin' antiangiogenic efficacy may be mediated through nitric oxide (NO) signaling which plays a major role in tumor angiogenesis. NO is a free radical that regulates diverse physiological and pathological events. Sustained and/or excess NO generation occurs during lung cancer development and progression (17). NO is synthesized by three major isoforms of NO synthase (NOS), constitutively expressed endothelial (eNOS), neuronal (nNOS) NOS and inducible NOS (iNOS). Of these isozymes, iNOS produces the most NO (18). More iNOS is expressed in lung tumors than in surrounding normal tissue, and iNOS levels are high in alveolar and tumor-associated macrophages, pulmonary endothelium, airway epithelium, and human AC (19). iNOS expression/activity correlates with angiogenic status and metastatic potential in a wide range of tumors, and iNOS inhibition by natural and synthetic compounds has been efficacious in cancer chemoprevention studies (20,21). Previously we hypothesized that dietary silibinin mediates its antiangiogenic effects by targeting iNOS in urethane-induced A/J mouse lung tumorigenesis (14).

Herein, we investigate the chemopreventive efficacy of silibinin on urethane-induced lung tumorigenesis in terms of tumor growth and progression in B6/129 wild-type (WT) mice and their iNOS^{-/-} counterparts. We hypothesized that if lung cancer development is regulated by NO production, silibinin should, at least in part, exert its chemopreventive effects through iNOS inhibition. The findings in the present study supported this hypothesis, and also highlighted, for the first time, the usefulness of micro-computed tomography (CT), a non-invasive imaging technique, to monitor real-time progression of lung tumors in evaluating the efficacy of silibinin.

Materials and Methods

Animals

Male B6/129-Nos2^{tm1Lau} (iNOS^{-/-}) and B6/129PF2 WT mice (5–6 weeks of age), from Jackson (Bar Harbor, ME) were injected i.p. with 1mg/g body weight urethane once weekly for 7 consecutive weeks (22). Eight weeks after the initial urethane injection, mice were randomly divided into two groups and gavaged with either 0.2 ml vehicle (0.5% w/v carboxy methyl cellulose and 0.025% Tween 20 in distilled water) or silibinin (742mg/kg body weight) in vehicle for 5 days/week for 18 weeks. This dose of silibinin corresponds to 1% w/w of silibinin in diet (14) and has been used in our previous study (15). Animal care and experiments were conducted in accordance with an IACUC-approved protocol at University of Colorado Denver. Twenty-five weeks after the first urethane injection, mice were euthanized, tumors enumerated, and tumor diameters measured using digital calipers. Lungs from 4 mice/group were perfused, formalin-fixed, and paraffin-embedded for histological and immunohistochemical analyses.

Non-invasive micro-CT imaging

After the final urethane injection, a subset (n=6) of mice from control and silibinin-treated groups underwent 4 monthly CT scans (at 0, 4, 8 and 12 weeks of silibinin treatment) for longitudinal assessment of number and diameter of lung lesions. Mice were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine mixture (i.p.), placed on a warming pad, and inserted into a Siemens Inveon micro-CT scanner (Siemens Preclinical Solutions, Knoxville, TN). A single 3-dimensional (3D) micro-CT image set was acquired for each mouse using following parameters: 270° rotation; 240 rotation steps; CCD read-out of 2304/2048; 4 binnings for matrix size reduction; exposure time of 30 ms with 80 kV voltage and 450 μ A current; with a field of view (FOV) of 34.68 $\times$ 30.83 mm. The 6-min acquisition with middle-to-high magnification resulted in effective isotropic resolution of 60 microns (after Shepp-Logan reconstruction algorithm). Animals were monitored during recovery from the anesthesia and returned to their cages. CT image reading was performed by an image specialist (NJS) who was blinded to animal group assignments. All post-processing analysis was performed using a high-speed CT reconstruction system (COBRA, Siemens Preclinical Solutions) and AsiPro image software for total lesion counts. Maximal diameters of each lesion were also determined for each anatomical direction (transaxial, coronal, and sagittal) and mean diameter determined. Motion artifacts were reduced by restraining anesthetized mice.

Immunohistochemical analysis and quantification of protein expression

Serial tissue sections (5 μ m) were sliced from paraffin-embedded formalin-fixed lungs and immunohistochemical staining performed as described previously (15). Briefly, tissue sections were deparaffinized, hydrated, and stained using specific primary antibodies, biotin-conjugated secondary antibodies, and horse radish peroxidase (HRP)-conjugated avidin. Specific antibody interactions were detected with the HRP substrate 3, 3'-diaminobenzidine (DAB). Primary antibodies used were mouse monoclonal anti-PCNA (1:250, from Dako, Carpinteria, CA), nestin (1:100 dilution, rabbit polyclonal anti-eNOS (1:100 dilution, nNOS (1:100 dilution), iNOS (1:100 dilution), pSTAT3 (Ser727; 1:200 dilution), and p65NF- κ B (Ser276; 1:100 dilution; all from Abcam, Cambridge, MA) followed by appropriate secondary antibodies (Vector Laboratories, Burlingame, CA). PCNA-, pSTAT3- and p65/NF- κ B-positive cells were quantified by counting the number of brown-stained nuclei/total number of cells in 5 randomly selected 400 $\times$ magnified fields. eNOS, nNOS and iNOS were quantified by immunoreactivity (represented by intensity of brown staining) and scored as 0 (no staining), +1 (very weak), +2 (weak), +3 (moderate), and +4 (strong) at 5 randomly selected 400 $\times$ magnified fields. Newly formed nestin-positive microvessels were quantified

as mean number of positive vessels in 5 randomly selected 400× magnified fields per tumor. All microscopic, histological, and IHC analyses were performed with a Zeiss Axioskop 2 microscope (Carl Zeiss, Inc.), and photomicrographs were captured with a Carl Zeiss AxioCam MRC5 camera.

Immunoblot analysis

Randomly selected frozen tumor samples from four individual mice from WT urethane and silibinin-treated groups were homogenized and lysates prepared as reported previously (15). Equal protein per lysate was denatured with 2× sample buffer and resolved on Tris-glycine gels, transferred onto nitrocellulose membrane, and blocked for 1h with 5% non-fat dry milk. Membranes were incubated with specific primary antibodies including anti-iNOS (BD BioSciences), anti-nestin and anti-VEGFR2 (Santa Cruz Biotechnology), followed by peroxidase- conjugated appropriate secondary antibody. These antibodies for immunoblot analyses provide better results. Finally, proteins were visualized by enhanced chemiluminescence detection. To confirm equal protein loading, membranes were stripped and re probed with mouse monoclonal anti-beta-actin primary antibody (Sigma) in each case.

Statistical analysis

Statistical analyses were performed using SigmaStat software version 3.5 (Jandel Scientific). Quantitative data are presented as mean ± SEM. Statistical significance of differences between control and silibinin-treated groups was determined by an unpaired Student's *t*-test, with $P<0.05$ considered statistically significant.

Results

Imaging analysis of lung tumors

Imaging technology has been used to monitor lung tumor progression and evaluate the efficacy of preventive/therapeutic approaches. Micro-CT provides 3D representations of high density tissue structures needed to monitor therapeutic effects over time. This optimizes treatment intervals and reduces the number of animals needed to get reliable results (23). Micro-CT is a preferred imaging modality of choice for lung lesions. Magnetic resonance imaging, (MRI) exhibits low signal intensity and susceptibility artifacts in lung (24). We monitored growth inhibitory effects of silibinin on urethane-induced lung tumorigenesis by subjecting control and silibinin-treated, tumor-bearing WT mice to micro-CT scanning at 4 week intervals. Micro-CT images clearly distinguished lung tumors from surrounding tissue even without any contrast agent, and the reconstructed 3D pulmonary images easily differentiated tumors from blood vessels (Fig. 1A). WT animals developed an average of 5, 7 and 9 tumors at 4, 8 and 12-weeks, respectively, whereas silibinin-treated animals developed only 2, 3, and 3 tumors, respectively, at the same time points (Fig. 1B). Average tumor diameter increased from 0.5 to 2 mm in control animals gradually over 12 weeks. Silibinin treatment significantly reduced tumor diameter by 43% ($P<0.02$) and 72% ($P<0.005$) at the 8th and 12th weeks compared to corresponding controls, respectively (Fig. 1C). These results directly correlate with our *ex vivo* tumor data measured at the end of the experiment (Fig. 2B), and confirm the applicability of micro-CT imaging as a non-invasive and quantitative, real-time monitoring tool of lung tumor progression. The calculation of lung lesions for each mouse in each CT session was performed in tri-plicate by an imaging scientist who was ignorant to the group assignment and histological findings. The inter-day variability for CT reads on number of lesions was 8% and on lesion diameters 14%.

Silibinin inhibits urethane-induced lung tumorigenesis in WT but not iNOS^{-/-} mice

Tumors were extracted from lungs, and tumor number and diameters compared between WT and iNOS^{-/-} mice. WT mice developed an average of 15 lung tumors/mouse, whereas iNOS^{-/-} animals developed only 2 lung tumors/mouse, an 87% ($P<0.001$) reduction in tumor multiplicity (Fig. 2A) in agreement with our earlier studies (25). Silibinin treatment significantly reduced lung tumor multiplicity by 71% ($P<0.01$) in WT mice, but there was no statistically-significant change in tumor number in control versus silibinin-treated iNOS^{-/-} mice (Fig. 2A). WT mice developed lung tumors with diameters varying from 0.5 mm to 4.5 mm, while all tumors from iNOS^{-/-} mice were <1 mm in diameter, an 82% reduction. Silibinin retarded tumor progression of the <1 mm lesions by 67% ($P<0.01$) and of the 1.0–1.5 mm lesions by 62%, and completely suppressed the progression to 1.5–2.5 mm ($P<0.05$) and >2.5 mm diameter lesions in WT mice. However, silibinin did not further reduce tumor size or number in iNOS^{-/-} mice (Fig. 2B). Due to small size and number of iNOS^{-/-} tumors, we were unable to use them for molecular analysis.

Histopathological characteristics of lung tissue and tumors

Histopathological examination of lung tissue and tumors in B6/129 mice 16 weeks after the last of seven weekly urethane injections yielded adenomas that exhibited uniform cellular organization, mild nuclear dysmorphology and a normal nuclear/cytoplasmic ratio. Airways and alveolar spaces immediately adjacent to the tumors were compressed slightly, and the tumors were surrounded by a small number of macrophages (data not shown). There were no significant differences in histopathology between lesions from silibinin-fed and control groups.

Silibinin selectively reduces the level of iNOS expression in urethane-induced lung tumors

Since in iNOS^{-/-} mice silibinin did not show any considerable effect on urethane-induced lung tumorigenesis, we anticipated that antitumor effect of silibinin could be mostly mediated *via* iNOS in WT mice. Therefore, we analyzed the levels of all the three forms of NOS in tumors. Immunohistochemical examination of iNOS, eNOS and nNOS levels in control and silibinin-treated tumor-bearing WT mice showed that silibinin decreased iNOS levels in tumors by 57% ($P<0.001$), which was also confirmed by immunoblot analysis (Fig. 3A). Silibinin did not show any considerable effects on eNOS and nNOS expression levels (Figs. 3B and 3C). These results provided evidence that silibinin targets iNOS for its antitumor activity in urethane-induced lung tumorigenesis of mouse model.

Silibinin inhibits cell proliferation and neoangiogenesis in urethane-induced lung tumors

Since we observed silibinin-mediated decreases in number and sizes of lung tumors, we next analyzed whether silibinin affected cell proliferation and angiogenesis in these tumors. Silibinin decreased expression of the proliferative marker, PCNA, by 18% ($P<0.01$) compared to controls (Fig. 4A). Microscopic examination of nestin, an angiogenic marker for newly formed microvessels, showed numerous nestin-positive microvessels and increased immunoreactivity in tumors of control mice. Silibinin decreased the number of nestin-positive tumor vessels by 61% ($P<0.01$), which was also confirmed by immunoblot analysis (Fig. 4B). We also observed a significant decrease (55%; $P<0.01$) in the intensity of nestin immunostaining in silibinin-treated versus control tumor-bearing WT mice (data not shown).

The growth of new blood vessels in tumors depends on signaling pathways regulated by vascular endothelial growth factor (VEGF) and its major receptor, VEGFR2 (26). VEGFR2 expression analysis by immunoblotting revealed a significant decrease ($P<0.01$) in the

silibinin-treated group compared to controls (Fig. 4C). These results suggest that silibinin-caused down-regulation of VEGFR2 could mediate its anti-neoangiogenesis in lung tumors.

Silibinin inhibits STAT3 and NF- κ B activation in urethane-induced lung tumors

The transcription factors, STAT3 and NF- κ B, play vital roles in tumor angiogenesis, and the promoter region of *iNOS* has binding sites for both STAT3 and NF- κ B (27). We determined STAT3 and NF- κ B activities by examining pSTAT3 (Ser727) and p65NF- κ B (Ser276) expression and localization in lung tumors by IHC. Silibinin treatment significantly decreased nuclear pSTAT3 (Ser727)-positive cells by 38% ($P<0.001$) compared to the control group of tumors (Fig. 5A). Similarly, silibinin also decreased nuclear p65NF- κ B (Ser276)-positive cells by 31% ($P<0.001$) as compared to the control group of tumors (Fig. 5B). These results indicate the inhibitory effect of silibinin on STAT3 and NF- κ B signaling in lung tumors which could have mediated its chemopreventive and angiopreventive effects most likely through iNOS regulation.

Discussion

The focus of the present study was to assess the chemopreventive potential of oral silibinin on multiple injections of urethane-induced lung tumors in B6/129 wild type and *iNOS*^{-/-} mice. Previous studies from our laboratory showed that silibinin inhibits tumor formation and the growth of advanced lung tumors in the A/J mouse model (14,15). We previously observed that silibinin inhibited iNOS and COX-2 expression, the two enzymes which promote lung tumor growth and progression in this model (14). Herein, we demonstrated the usefulness of micro-CT for evaluating the chemopreventive efficacy of silibinin on urethane-induced lung tumorigenesis. Micro-CT images revealed that the number of tumors increased over time after carcinogen exposure, and that silibinin treatment inhibited this growth. We determined that the total number of tumors detected by CT (extrapolated for 18 weeks) was slightly lower than that detected by macroscopic study at 18 weeks, suggesting that micro-CT could not detect all tumors in this study. This may be explained by the differences in spatial resolution of a macroscopic imaging technique in the living animal versus microscopic histological examination of tissues. However, the trends correlated well and further improvement of the micro-CT imaging protocol is under development. Our results also support recent findings that micro-CT can detect lung cancer lesions as small as 1-mm diameter with high resolution (28–31). Haines *et al.* (30) longitudinally demonstrated that micro-CT could be used to study lung cancer progression and treatment response to erlotinib in mice genetically-engineered to produce mutant *KRAS* driven lung tumors. Fushiki *et al.* (29) described a strong correlation between bioluminescence and micro-CT imaging in early lung lesion detection and quantitatively assessed cancer response to cisplatin and gemcitabine therapy in an orthotopic model. Wang *et al.* (31) showed that both MRI and micro-CT can differentiate squamous cell carcinoma from adenocarcinoma induced by N-nitroso-trischloroethylurea. We quantitatively assessed silibinin efficacy in carcinogen-treated, genetically-modified mice and demonstrated a strong correlation between histopathological endpoints and real-time quantitative micro-CT.

To determine whether silibinin's chemopreventive activity was working through iNOS, mice lacking the iNOS gene were given a multiple urethane carcinogenesis regimen. We have previously determined that this regimen induces a high multiplicity and incidence of lung tumors in the moderately resistant B6/129 strain (25). This study demonstrates that silibinin is an effective chemopreventive agent for lung tumor formation in strains other than A/J injected once with urethane as compared with a more aggressive carcinogenesis protocol employing multiple injections of urethane. Eighteen weeks of silibinin administration reduced lung tumor multiplicity as well as size, and this was accompanied by significant anti-angiogenic effects. B6 resistance alleles present in these chimeric mice did not affect

silibinin efficacy, suggesting that silibinin exerts its chemopreventive efficacy on lung tumorigenesis irrespective of genetic background. The 87% decrease in tumor number in *iNOS*^{-/-} mice confirms that *iNOS* is an important mediator of lung tumorigenesis in this model. Since silibinin did not considerably affect either tumor number or size in *iNOS*^{-/-} mice, these mice suggest that silibinin exerts its chemopreventive effects mostly through inhibiting *iNOS* expression in lung tumors.

To further explore the mechanisms of lung cancer chemoprevention by silibinin, we evaluated the effect of silibinin on cancer cell proliferation. Cell proliferation was assessed by PCNA expression, a cofactor of DNA polymerase required for DNA replication and nucleotide excision repair (32). We observed that lung tumors from silibinin-treated mice had significantly lower PCNA expression than their urethane-treated controls, suggesting that at least part of silibinin's mechanism of chemoprevention is through inhibition of tumor cell proliferation.

Neo-angiogenesis facilitates tumor growth, progression and metastasis. Newly formed microvessels were observed in urethane-induced lung tumors in WT mice, and silibinin treatment significantly decreased the number of these vessels. Since VEGF and its receptors are highly expressed in many tumors (33,34), *iNOS* expression is associated with increased VEGF expression, and VEGF expression is decreased in the lung tumors of *iNOS*-deficient mice (25), we analyzed the expression of angiogenic factors in tumors from control and silibinin-treated mice. VEGFR2 expression level and nestin immunoreactivity were decreased in silibinin-treated mice, implying that angiopreventive mechanisms of silibinin are related to *iNOS*-dependent suppression of vascular growth in lung tumors.

Next we evaluated the activation state of STAT3 and NF- κ B, transcription factors which play major roles in tumorigenesis of a wide variety of cancers including lung cancer (35,36). STAT3 nuclear translocation and activation is known to increase expression of genes which influence cell cycle progression (such as cyclin D1) and angiogenesis (such as VEGF) (35). Other studies have demonstrated the importance of NF- κ B signaling in chemically-induced and genetic models of lung cancer (37,38). In the present study, silibinin strongly inhibited the activation of STAT3 and NF- κ B in multiple urethane-induced lung tumors of WT mice. This is further supported by our earlier observations where silibinin inhibited macrophage infiltration as well as the activation of STAT3 and NF- κ B in tumors from urethane-treated A/J mice, and has also inhibited doxorubicin-induced NF- κ B activation in A549 cells (12,15).

Nitric oxide production by *iNOS* is involved in the pathogenesis of many cancers. When we analyzed *iNOS* expression by immunohistochemistry and immunoblotting in WT mice, we found that silibinin inhibited *iNOS* expression without affecting that of eNOS and nNOS. High *iNOS* levels are shown in lung large-cell carcinoma and lung adenocarcinoma but not in squamous cell carcinoma of human non-small cell lung cancer (39). *iNOS*/NO levels are higher in lung tumors from smokers than non-smokers, and in human lung tumors compared to surrounding tissue (17,40). Inhibition of *iNOS* activity reduces aberrant crypt foci formation and colon tumor formation (41). Silibinin is shown to inhibit *iNOS* expression and cytokine-induced activation of STAT and NF- κ B in non-small cell lung cancer A549 cells (12). Since the *iNOS* gene promoter region has binding sites for STAT3 and NF- κ B, the inhibition of the activation of these transcription factors by silibinin may account for the observed decrease in *iNOS* expression (27).

In summary, we demonstrate the chemopreventive efficacy of silibinin in the B6/129 WT lung tumorigenesis model. The key findings are: (a) *iNOS*^{-/-} mice developed few tumors, indicating the importance of *iNOS* in lung tumorigenesis; (b) silibinin decreased the number

and size of tumors in B6/129 WT but not in iNOS^{-/-} mice; (c) these decreases in tumor number and size correlate with silibinin-mediated inhibition of cell proliferation and angiogenesis in WT mice, most likely through the inhibition of iNOS expression involving down-regulation of STAT and NF- κ B signaling; and (d) micro-CT analysis represents a sensitive and reliable imaging modality to longitudinally assess the inhibition of growth of lung tumors by chemopreventive agents including silibinin, and therefore could also be useful in other lung cancer intervention studies.

Statement of Translational Relevance

Overexpression of inducible nitric oxide synthase (iNOS) and sustained nitric oxide (NO) generation positively correlate with lung cancer development and progression. This study genetically confirmed this role of iNOS using urethane-induced lung tumorigenesis in B6/129-Nos2^{tm1Lau} (iNOS^{-/-}) and B6/129PF2 WT mice. This study also showed that silibinin, a cancer chemopreventive agent, decreases lung tumor multiplicity by 71% ($P<0.01$) in WT mice, but failed to have such efficacy in iNOS^{-/-} mice, suggesting iNOS as a potential chemopreventive target during lung carcinogenesis. The findings also showed that silibinin exerts its chemopreventive and angiopreventive effects mostly through its inhibition of iNOS expression involving down-regulation of STAT3 and NF- κ B signaling, which otherwise are up-regulated in lung tumors. Overall, these results support iNOS as a potential target for controlling lung cancer by silibinin, and demonstrate the application of real-time non-invasive micro-CT imaging for evaluation of the efficacy of lung cancer chemopreventive agents.

Acknowledgments

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Abbreviations

iNOS	inducible nitric oxide
NSCLC	non-small cell lung cancer
NO	nitric oxide
CT	computed tomography
MRI	Magnetic resonance imaging

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin*. 2009; 59:225–249. [PubMed: 19474385]
2. Krol AD, Aussems P, Noordijk EM, Hermans J, Leer JW. Local irradiation alone for peripheral stage I lung cancer: could we omit the elective regional nodal irradiation? *Int J Radiat Oncol Biol Phys*. 1996; 34:297–302. [PubMed: 8567329]
3. Beitler JJ, Badine EA, El-Sayah D, et al. Stereotactic body radiation therapy for nonmetastatic lung cancer: an analysis of 75 patients treated over 5 years. *Int J Radiat Oncol Biol Phys*. 2006; 65:100–106. [PubMed: 16517092]
4. Gu M, Singh RP, Dhanalakshmi S, Agarwal C, Agarwal R. Silibinin inhibits inflammatory and angiogenic attributes in photocarcinogenesis in SKH-1 hairless mice. *Cancer Res*. 2007; 67:3483–3491. [PubMed: 17409458]

5. Dhanalakshmi S, Mallikarjuna GU, Singh RP, Agarwal R. Dual efficacy of silibinin in protecting or enhancing ultraviolet B radiation-caused apoptosis in HaCaT human immortalized keratinocytes. *Carcinogenesis*. 2004; 25:99–106. [PubMed: 14555614]
6. Singh RP, Raina K, Deep G, Chan D, Agarwal R. Silibinin suppresses growth of human prostate carcinoma PC-3 orthotopic xenograft via activation of extracellular signal-regulated kinase 1/2 and inhibition of signal transducers and activators of transcription signaling. *Clin Cancer Res*. 2009; 15:613–621. [PubMed: 19147767]
7. Singh RP, Raina K, Sharma G, Agarwal R. Silibinin inhibits established prostate tumor growth, progression, invasion, and metastasis and suppresses tumor angiogenesis and epithelial-mesenchymal transition in transgenic adenocarcinoma of the mouse prostate model mice. *Clin Cancer Res*. 2008; 14:7773–7780. [PubMed: 19047104]
8. Roy S, Kaur M, Agarwal C, Tecklenburg M, Sclafani RA, Agarwal R. p21 and p27 induction by silibinin is essential for its cell cycle arrest effect in prostate carcinoma cells. *Mol Cancer Ther*. 2007; 6:2696–2707. [PubMed: 17938263]
9. Flaig TW, Su LJ, Harrison G, Agarwal R, Glode LM. Silibinin synergizes with mitoxantrone to inhibit cell growth and induce apoptosis in human prostate cancer cells. *Int J Cancer*. 2007; 120:2028–2033. [PubMed: 17230508]
10. Singh RP, Tyagi A, Sharma G, Mohan S, Agarwal R. Oral silibinin inhibits in vivo human bladder tumor xenograft growth involving down-regulation of survivin. *Clin Cancer Res*. 2008; 4:300–308. [PubMed: 18172282]
11. Rajamanickam S, Velmurugan B, Kaur M, Singh RP, Agarwal R. Chemoprevention of intestinal tumorigenesis in APCmin/+ mice by silibinin. *Cancer Res*. 2010; 70:2368–2378. [PubMed: 20215518]
12. Chittezhath M, Deep G, Singh RP, Agarwal C, Agarwal R. Silibinin inhibits cytokine-induced signaling cascades and down-regulates inducible nitric oxide synthase in human lung carcinoma A549 cells. *Mol Cancer Ther*. 2008; 7:1817–1826. [PubMed: 18644994]
13. Singh RP, Mallikarjuna GU, Sharma G, et al. Oral silibinin inhibits lung tumor growth in athymic nude mice and forms a novel chemocombination with doxorubicin targeting nuclear factor kappaB-mediated inducible chemoresistance. *Clin Cancer Res*. 2004; 10:8641–8647. [PubMed: 15623648]
14. Singh RP, Deep G, Chittezhath M, et al. Effect of silibinin on the growth and progression of primary lung tumors in mice. *J Natl Cancer Inst*. 2006; 98:846–855. [PubMed: 16788158]
15. Tyagi A, Singh RP, Ramasamy K, et al. Growth inhibition and regression of lung tumors by silibinin: modulation of angiogenesis by macrophage-associated cytokines and nuclear factor-kappaB and signal transducers and activators of transcription 3. *Cancer Prev Res (Phila Pa)*. 2009; 2:74–83.
16. Mateen S, Tyagi A, Agarwal C, Singh RP, Agarwal R. Silibinin inhibits human nonsmall cell lung cancer cell growth through cell-cycle arrest by modulating expression and function of key cell-cycle regulators. *Mol Carcinog*. 2010; 49:247–258. [PubMed: 19908243]
17. Fujimoto H, Ando Y, Yamashita T, et al. Nitric oxide synthase activity in human lung cancer. *Jpn J Cancer Res*. 1997; 88:1190–1198. [PubMed: 9473737]
18. Murakami A. Chemoprevention with phytochemicals targeting inducible nitric oxide synthase. *Forum Nutr*. 2009; 61:193–203. [PubMed: 19367123]
19. Liu CY, Wang CH, Chen TC, Lin HC, Yu CT, Kuo HP. Increased level of exhaled nitric oxide and up-regulation of inducible nitric oxide synthase in patients with primary lung cancer. *Br J Cancer*. 1998; 78:534–541. [PubMed: 9716040]
20. Murakami A. Chemoprevention with phytochemicals targeting inducible nitric oxide synthase. *Nitric Oxide*. 2008; 19:217–224. [PubMed: 18515106]
21. Fitzpatrick B, Mehibel M, Cowen RL, Stratford IJ. iNOS as a therapeutic target for treatment of human tumors. *Forum Nutr*. 2009; 61:193–203. [PubMed: 19367123]
22. Malkinson AM, Beer DS. Major effect on susceptibility to urethan-induced pulmonary adenoma by a single gene in BALB/cBy mice. *J Natl Cancer Inst*. 1983; 70:931–936. [PubMed: 6573537]
23. De Clerck NM, Meurrens K, Weiler H, et al. High-resolution X-ray microtomography for the detection of lung tumors in living mice. *Neoplasia*. 2004; 6:374–379. [PubMed: 15256059]

24. Schuster DP, Kovacs A, Garbow J, Piwnica-Worms D. Recent advances in imaging the lungs of intact small animals. *Am J Respir Cell Mol Biol.* 2004; 30:129–138. [PubMed: 14729505]
25. Kisley LR, Barrett BS, Bauer AK, et al. Genetic ablation of inducible nitric oxide synthase decreases mouse lung tumorigenesis. *Cancer Res.* 2002; 62:6850–6856. [PubMed: 12460898]
26. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med.* 2003; 9:669–676. [PubMed: 12778165]
27. Kleinert H, Wallerath T, Fritz G, et al. Cytokine induction of NO synthase II in human DLD-1 cells: roles of the JAK-STAT, AP-1 and NF-kappaB-signaling pathways. *Br J Pharmacol.* 1998; 125:193–201. [PubMed: 9776360]
28. Johnson EM, Price RE, Kurie JM, Rivera BS, Cody DD. A new method for respiratory gating during microcomputed tomography of lung in mice. *J Am Assoc Lab Anim Sci.* 2008; 47:46–56. [PubMed: 18702451]
29. Fushiki H, Kanoh-Azuma T, Katoh M, et al. Quantification of mouse pulmonary cancer models by microcomputed tomography imaging. *Cancer Sci.* 2009; 100:1544–1549. [PubMed: 19459854]
30. Haines BB, Bettano KA, Chenard M, et al. Quantitative Volumetric Micro-Computed Tomography Method to Analyze Lung Tumors in Genetically Engineered Mouse Models. *Neoplasia.* 2009; 11:39–47. [PubMed: 19107230]
31. Wang Y, Zhang Z, Garbow JR, et al. Chemoprevention of lung squamous cell carcinoma in mice by a mixture of Chinese herbs. *Cancer Prev Res (Phila Pa).* 2009; 2:634–640.
32. Bacchi CE, Gown AM. Detection of cell proliferation in tissue sections. *Braz J Med Biol Res.* 1993; 26:677–687. [PubMed: 7903573]
33. Huss WJ, Hanrahan CF, Barrios RJ, Simons JW, Greenberg NM. Angiogenesis and prostate cancer: identification of a molecular progression switch. *Cancer Res.* 2001; 61:2736–2743. [PubMed: 11289156]
34. Turner HE, Harris AL, Melmed S, Wass JA. Angiogenesis in endocrine tumors. *Endocr Rev.* 2003; 24:600–632. [PubMed: 14570746]
35. Haura EB, Turkson J, Jove R. Mechanisms of disease: insights into the emerging role of signal transducers and activators of transcription in cancer. *Nat Clin Pract Oncol.* 2005; 2:315–324. [PubMed: 16264989]
36. Lin, Yong; Bai, Lang; Chen, Wenjie; Xu, Shanling. The NF-kappaB activation pathways, emerging molecular targets for cancer prevention and therapy. *Expert Opin Ther Targets.* 2010; 14:45–55. [PubMed: 20001209]
37. Stathopoulos GT, Sherrill TP, Cheng DS, et al. Epithelial NF-kappaB activation promotes urethane-induced lung carcinogenesis. *Proc Natl Acad Sci U S A.* 2007; 104:18514–18519. [PubMed: 18000061]
38. Takahashi H, Ogata H, Nishigaki R, Broide DH, Karin M. Tobacco smoke promotes lung tumorigenesis by triggering IKKbeta- and JNK1-dependent inflammation. *Cancer Cell.* 2010; 17:89–97. [PubMed: 20129250]
39. Marrogi AJ, Travis WD, Welsh JA, et al. Nitric oxide synthase, cyclooxygenase 2 and vascular endothelial growth factor in the angiogenesis of non-small cell lung carcinoma. *Clin Cancer Res.* 2000; 64:4739–4744. [PubMed: 11156228]
40. Chen GG, Lee TW, Xu H, et al. Increased inducible nitric oxide synthase in lung carcinoma of smokers. *Cancer.* 2008; 112:372–381. [PubMed: 18008356]
41. Ahn B, Ohshima H. Suppression of intestinal polyposis in Apc(Min/+) mice by inhibiting nitric oxide production. *Cancer Res.* 2001; 61:8357–8360. [PubMed: 11731407]

Figure 1.

Micro-CT images and quantitative end-points for lung tumor growth and chemopreventive effect of silibinin. **A**, Axial micro-CT images of tumor growth in urethane and urethane + silibinin treated WT mice at the indicated weeks after silibinin treatment. Tumors observed in the lungs are circled. Quantitative analysis of tumor number (**B**) and diameter (**C**) by micro-CT imaging. Each tumor scanned by micro-CT was reconstructed to three-dimensional images (axial, sagittal and coronal), and diameters measured periodically as indicated. Data represent mean $\pm$ SEM value of six animals in each group. Sb, silibinin.

Figure 2.

Silibinin prevents urethane-induced lung tumor growth and progression in WT but not in *iNOS*^{-/-} mice. *A*, Lung tumors were dissected from all groups and tumor multiplicity/mouse determined. *B*, Tumor diameters were measured with digital calipers under a dissecting microscope and grouped by diameter: <1.0 mm, 1.0–1.5 mm, >1.5 mm–2.5 mm and >2.5 mm. Data represent mean $\pm$ SEM value from tumors of four animals in each group. Sb, silibinin; iNOS KO, iNOS knock out.

Figure 3.

Silibinin selectively decreases iNOS expression in lung tumors. Lung tumors from WT mice were processed for IHC staining and quantification for: *A*, iNOS; *B*, eNOS; and *C*, nNOS. IHC quantitative data represent mean $\pm$ SEM of four animals in each group. *A*, lower panel, iNOS levels were also analyzed by Western blot in four randomly-selected tumor samples from four individual animals in control and silibinin-treated WT mice. Sb, silibinin.

Figure 4.

Silibinin inhibits proliferation and neo-angiogenesis in lung tumors. Lung tumors were immunohistochemically analyzed for: *A*, PCNA; and *B*, nestin, and quantified for PCNA-positive cells and nestin-positive microvessels (*arrows*, nestin-positive vessels), respectively, as detailed in Materials and Methods. Four randomly-selected tumor samples from four individual animals in control and silibinin-treated WT mice were analyzed by Western blot for: *B*, lower panel, Nestin and *C*, VEGFR2 expression levels. IHC Quantitative data for PCNA and nestin represent the mean $\pm$ SEM value of four animals; quantitative densitometric data for VEGFR2 represent the mean $\pm$ SEM value of four bands in each group. Sb, silibinin.

Figure 5.

Silibinin decreases the activation of STAT3 and p65NF-κB in lung tumors. Lung tumors from WT mice were processed for IHC staining and quantified for: *A*, pSTAT3 (Ser727) and *B*, p65NF-κB (Ser276) using phospho-specific primary antibodies as detailed in Materials and Methods. IHC quantitative data in each case represent the mean $\pm$ SEM of four animals in each group. Sb, silibinin.